

British Journal of Pharmacy

www.bjpharm.hud.ac.uk

Critical Review

Circulating Tumour DNA: a Minimally Invasive Biomarker for Tumour Detection and Stratification

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ARTICLE INFO

 Received: 30/06/2016
 Revised: 08/09/2016
 Accepted: 14/10/2016
 Published: 14/11/2016

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KEYWORDS:

 Circulating tumour DNA,
 Liquid biopsy, Biomarker,
 Tumor detection, Tumor
 stratification

ABSTRACT

Genetic and epigenetic alterations significantly contribute to development of human cancer. Genotyping tumour tissue in search for these actionable genetic and epigenetic changes has become routine practice in oncology. However, sampling tumour tissue has significant inherent limitations. It provides only a single snapshot in time, prone to selection bias due to intra-tumour heterogeneity, and cannot always be performed owing to its invasive nature. Circulating tumour DNA (ctDNA) based liquid biopsy provides an effective alternative to invasive tissue sampling and have emerged as a minimally invasive, real-time biomarker. Recent advancements in DNA sequencing technologies have revealed enormous potential of ctDNA to improve tumour detection and stratification. In this review, we critically appraise the role of ctDNA as a liquid biopsy for cancer and evaluate the role of circulating tumour DNA as a diagnostic, prognostic and predictive biomarker. We also highlight some technical challenges and constraints associated with circulating DNA analysis.

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INTRODUCTION

The advent of personalised medicine has revolutionised cancer management. Advancements in genomics and transcriptomics have enabled identification of gene signatures and targets that can be used for diagnostics, prognostics, predictive and therapeutic purposes (Gonzalez de Castro et al. 2013). Analysis of tumour-linked genetic alterations has become routine practice in oncology. Genetic profiling of solid tumours requires the sample of the abnormal tissue (mass, lesions or tumour) which is usually obtained by invasive biopsy (Amalou and Wood 2012). However, the invasive tissue biopsy is associated with significant risks, complications and limitations described below.

Intra-tumour heterogeneity and tumour evolution

It has been shown that a tumour consists of a variety of sub-clones that harbour different genetic and epigenetic alterations, a phenomena termed as intra-tumour heterogeneity (ITH) (Marusyk et al. 2012; Swanton 2012). A study by Gerlinger et al. (2012) showed that biopsy samples taken from different regions of Renal Cell Carcinoma displayed heterogeneous somatic mutations and were not detectable in every region of the tumour sequenced. Similarly, Bashashati et al. (2013) have observed regional diversity in mutations, copy numbers and gene expression profiles in primary ovarian cancers prior to therapeutic intervention. Therefore, biopsy of a small region of tumour might not account for ITH and could not accurately characterise distinct molecular alterations present within a single tumour.

It is also widely accepted that tumour development follows a process of Darwinian-like clonal evolution (Gerlinger and Swanton 2010; Gerlinger et al. 2012). High throughput sequential analysis of tumour samples has revealed that ITH evolves temporally under selection pressures imposed by the microenvironment and/or the cancer therapeutics (Anderson et al. 2006; Hunter et al. 2006; Edwards et al. 2008). It implies that the historical tissue biopsy taken at the time of diagnosis might not effectively guide clinical decisions after some passage of time and should be supplemented by serial tissue sampling to account for new mutations.

Limitations in tissue processing

Processing of tumour tissue sample affects the quality of DNA obtained. Studies have shown that formalin fixation causes DNA denaturation, base alteration and production of sequence artefact (Douglas and Rogers 1998; Oh et al. 2015). Gallegos Ruiz and colleagues (2007) have observed mutational artefacts in epidermal growth factor receptor (*EGFR*) gene resulting from formalin fixation and paraffin embedding of lung tissue sample. Therefore, formalin-processed tissue specimens might not accurately reflect the genetic alterations present in the tumour tissue. The use of fresh frozen tissue for processing might overcome these limitations, but it is restricted by logistic issues such as availability of liquid nitrogen and requirement of specialised equipment for processing and storage of frozen tissue (Budczies et al. 2011; Oh et al. 2015).

Associated risks and complications

Tissue sampling is invasive and is associated with significant risks and complications depending on the type and site of the biopsy. A systematic review on complications of the prostate biopsy by Loeb et al. (2013) showed that it is associated with complications such as pain, haematuria, haematospermia and urinary tract infection. Although most of these complications were mild and self-limiting, longer hospital stay and serious adverse events were reported in patients undergoing surgical biopsy (Lebofsky et al. 2015). Additionally, it is speculated that certain biopsies are associated with dislocation and seeding of malignant cells along the needle track leading to local recurrence and/or systemic spread (Shyamala et al. 2014).

Liquid biopsy - a paradigm shift in cancer testing:

The liquid biopsy is a minimally invasive blood test that detects tumour biomarkers (DNA, RNA, protein) in the circulation. It has emerged as an effective alternative to traditional tumour tissue sampling (Karachaliou et al. 2015). Being a simple blood test, it can be repeated to account for the changing genomic landscape of a tumour to track tumour evolution and monitor response to therapy. Additionally, it has the potential to interrogate genetic profiles of primary and metastatic tumours in the body which overcome intra-tumour heterogeneity and sampling bias. One of the most promising application of liquid biopsy is cell-free circulating tumour DNA (ctDNA).

THE BIOLOGY OF ctDNA

ctDNA, as the name implies, is the presence of tumour-DNA fragments in the circulation that shows similar molecular alterations present in the corresponding tumour in the body. The presence of cell-free nucleic acids in the human blood was first reported by Mandel and Metais (1948). It was subsequently found that the concentration of cell-free DNA is significantly higher in the circulation of cancer patients compared to healthy controls (Leon et al. 1977). This pioneering seminal work did not attract much interest until Stroun and colleagues (1989) showed that circulating DNA demonstrate similar properties such as decrease strand stability that were found in neoplastic cells. Subsequently, several proof of concept studies showed various tumour specific aberrations in the circulation such as mutation in oncogene and tumour suppressor gene (Sorenson et al. 1994; Silva et al. 1999b), microsatellite instability (Chen et al. 1996) and aberrant DNA methylation (Silva et al. 1999a). In recent years, studies are directed towards evaluating the clinical utility of ctDNA in the management of cancer (Takai et al. 2015).

Origin of ctDNA

ctDNA is the proportion of cell-free DNA (cfDNA) that originates from tumour cells and carry tumour-specific alterations. They are short fragments of double stranded DNA that measures between 70 to 200 base pairs but larger fragments of up to 21 kilobases were also recorded (Jahr et al. 2001). The percentage of cfDNA that is derived from tumour varies from patient to patient, ranging from as low as

0.01% to a mutant allelic fraction as high as 93% (Jahr et al. 2001; Diehl et al. 2005; Forsheew et al. 2012). It has been estimated that a patient with a tumour load of 100g releases up to 3.3% of tumour-DNA into the circulation on a daily basis (Diehl et al. 2005). Despite numerous studies describing cfDNA, the mechanism of the origin of these circulating DNA in the blood remains ambiguous. Two possible, mutually non-exclusive, mechanisms by which cfDNA enters into the circulation include passive release and active secretion (Figure 1). The passive release is mediated through the cellular destruction (both healthy and tumour cells) by apoptosis and necrosis (Jahr et al. 2001). Additionally, cells may actively secrete DNA into the blood in the form of nucleoprotein complex (Stroun et al. 2001). A low proportion of cfDNA is also contributed by the lysis of circulating tumour cells that have been shed from the primary tumour (Stroun et al. 2000).

Controversy exists in the literature regarding the contribution of necrosis and apoptosis in the origin of ctDNA. Some studies advocate that necrotic cells engulfed by the macrophages are the major source of ctDNA fragments (Diehl et al. 2005). However, recent studies have shown that these larger necrotic-derived DNA fragments were barely detectable in patients with pancreatic cancer (Sikora et al. 2015). Alternatively, many studies have suggested apoptosis as the main driver of release (reviewed in Vietsch et al. 2015). These observations were supported by the fact that ctDNA shows fragmentation pattern similar to nucleosomal DNA (~ 180 bp) (reviewed in Heitzer et al. (2015). Further studies are therefore required to elaborate the complex mechanism of origin of ctDNA and contribution of necrosis and apoptosis.

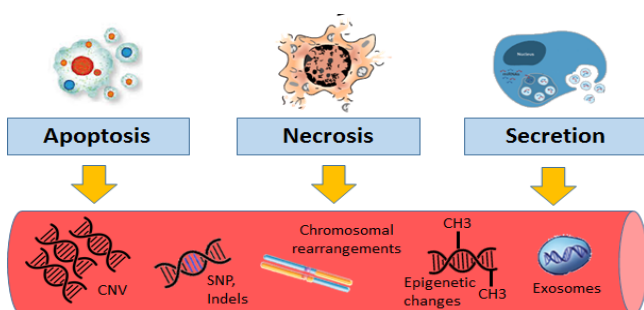


Fig 1: Mechanisms of release of cfDNA from tumour cells. Cancer-associated genetic alterations such as single nucleotide variants, copy number variations, methylation changes and chromosomal rearrangements can be detected in ctDNA

Tumourigenic potential of ctDNA: The Genometastasis Hypothesis

Interestingly, studies have shown that tumour-derived circulating DNA might transfect susceptible cells in the distinct target organs resulting in metastases. This hypothesis was proposed by García-Olmo and García-Olmo (2001) and have been supported by a number of experimental studies. A study by Garcia-Olmo et al. (2010) have demonstrated the potential of plasma DNA to transfect and oncogenically transform susceptible cells, and showed that plasma of colon cancer patient selectively transforms NIH/3T3 mouse cells while having no effect on human adipose-derived stem cells. Similar findings were also reported by Trejo-Becerril et al. (2012) in an *in vivo* immunocompetent colon-carcinogenesis rat model. However, a number of observations have limited the validity of this hypothesis. In the studies cited above plasma was used as the source of ctDNA. Studies have shown that plasma also contains different types of cell-derived particles carrying nucleic acids, for example, exosome, apoptotic bodies and microvesicles (Belting and Wittrup 2008), and they have been shown to play an important role in tumorigenesis (Azmi et al. 2013; Martins et al. 2013). Therefore, the oncogenic transformations observed by authors could have been mediated by these extracellular cell-derived particles as opposed to cell-free nucleic acid. Moreover, the organ-specific tropism of metastases could not be explained by this hypothesis (Hunter et al. 2008).

Kinetics of ctDNA

Little is known about the elimination of circulating DNA from blood. Studies on circulating foetal DNA from maternal plasma after delivery displays a half-life between 4 and 30 minutes with an initial rapid phase followed by a slower phase of clearance (Lo et al. 1999). Different studies have speculated that the clearance is mainly mediated by liver, kidney, spleen and other physiological factors in blood such as plasma deoxyribonuclease (Botezatu et al. 2000; Minchin et al. 2001; Cherepanova et al. 2007). However, the contribution of these mechanisms in the clearance of ctDNA is still controversial. A study by Lo et al. (1999) showed that plasma nucleases plays a limited role in the clearance of circulating foetal DNA from maternal plasma. On the contrary, other studies

have identified leading contribution of plasma nucleases and showed that low DNase activity in the plasma of cancer patients resulted in the high level of circulating DNA (Tamkovich et al. 2006; Cherepanova et al. 2008).

DETECTION OF ctDNA IN BLOOD

Methodological aspects

Two approaches are used to analyse circulating DNA as a biomarker for carcinogenesis: quantitative analysis of cfDNA and detection of tumour-specific alterations.

Quantitative analysis of total cfDNA

Studies have shown that the concentration of cfDNA is significantly higher in cancer patients compared to healthy controls owing to increased release from tumour cell turnover (Chang et al. 2002; Kamat et al. 2010). Review of published articles by van der Vaart and Pretorius (2010) reported that yields of circulating DNA in the plasma of cancer patients were on average 137 ng/ml, which is nine times greater than the concentration observed in the healthy individual (average 15 ng/ml).

Various methods are available to purify circulating DNA from serum or plasma. The classic DNA isolation procedure is based on phenol-chloroform method, a liquid-liquid extraction method that form a biphasic emulsion with aqueous layer containing DNA and organic layer containing precipitated proteins (Sengüven et al. 2014). Other commercially available kits using silica based technology such as Purelink Genomic DNA extraction kit (Invitrogen), and magnetic separation such as Agencourt DNAdvance Kit (Beckman Coulter) are also available (as reviewed in Dhaliwal 2013). The isolated DNA is then quantified using different techniques, including DNA dipsticks (Invitrogen), nick translation, qPCR and fluorometry with SYBR green (Xue et al. 2006; van der Vaart and Pretorius 2010).

Studies have demonstrated that quantitative analysis of cfDNA facilitates early detection of prostate, lung and breast cancer (Sozzi et al. 2003; Altimari et al. 2008; Kohler et al. 2009). A study by Sozzi et al. (2003) has shown eight-fold increase in the concentration of cfDNA in lung cancer patient compared to controls. However, conflicting results were observed in

different studies. In a study by Schmidt et al. (2008), no significant differences were observed in the amount of cfDNA in the tumour and non-tumour populations. Additionally, the concentration of cfDNA is also influenced by various physiological and pathological conditions such as pregnancy, exercise, heavy smoking, trauma, inflammatory diseases, premalignant conditions and chronic illnesses in elderly (reviewed in Fleischhacker and Schmidt 2007; Heitzer et al. 2015). Therefore, simple quantitative assessment has limited potential as a standalone biomarker, but it can be used as an adjunct with other conventional markers.

Characterising tumour-specific alterations in circulating DNA

Detecting tumour-specific genetic aberrations is a more sensitive and specific biomarker compared to the quantitative measurement of cfDNA (Jung et al. 2010). These genetic alterations include point mutations, insertions and deletions, multi-nucleotide polymorphisms, loss of heterozygosity, microsatellite alterations, copy number variations, chromosomal rearrangements and epigenetic alterations (reviewed in Fleischhacker and Schmidt 2007; Chen et al. 2016) (Figure 1).

Different technological platforms are available to detect these alterations. Broadly, they can be divided into two group i.e. targeted and untargeted approaches. The former includes the detection of known genetic changes in circulating DNA based on primary tumour genotype or analysing frequently occurring “driver” mutations in particular cancer. The untargeted approach includes techniques which do not require any prior knowledge of the mutation type. Techniques employed by different studies to detect ctDNA are summarised in Table 1.

The targeted approach is mainly based on PCR-based assays. Earlier techniques employed for detecting known minority (mutant) alleles mainly focused on allele specific PCR-based assays (AS-PCR) also called amplification-refractory mutation system (ARMS) (Reviewed in Diaz and Bardelli 2014). Although this technique is comparatively inexpensive and does not require any specialised instruments, the results of AS-PCR are semi-quantitative and provide only relative quantification to the control (Luke et al. 2014). This limitation has been overcome by combining AS-PCR

with quantitative methods, for example, fluorescently labelled primer and probe (Taqman, scorpion-ARMS) and peptide nucleic acid (PNA) which have improved interpretation of PCR results (Board et al. 2008). Other modifications of PCR-based assays have also been reported such as co-amplification at lower denaturation temperature-PCR (COLD-PCR) and competitive allele-specific hydrolysis probes

(TaqMan) PCR (CAST-PCR) (Table 1). Although AS-PCR and its different technical modifications are sensitive in detecting mutant DNA, cross-reactivity has been observed between the probes. In a study by Didelot et al. (2012) using CAST-PCR, cross-reactivity was observed between mutant and wild-type probes for *EGFR* resistant mutation (T790M) and it impacted assay's sensitivity.

Table 1. Overview of selected techniques used for detection of ctDNA.

Technique	Sensitivity ^a	Tumour type	Gene assessed for mutations	Selected studies using the technique
Allele-specific PCR	0.5% - 1%	NSCLC Breast CRC	<i>EGFR</i> <i>PIK3CA</i> <i>KRAS, BRAF</i>	(Kimura et al. 2006; Maheswaran et al. 2008; Goto et al. 2012) (Board et al. 2010) (Spindler et al. 2012)
Cold-PCR	0.01% - 0.1%	NSCLC CRC	<i>EGFR</i> <i>KRAS, BRAF</i>	(Li et al. 2008) (Mancini et al. 2010)
CAST-PCR	0.1-1 %	Melanoma Ovarian	<i>BRAF</i> <i>Beta-globin</i>	(Ashida et al. 2016) (Kamat et al. 2010)
PNA- PCR	0.1%-1%	CRC Pancreatic	<i>KRAS</i> <i>KRAS</i>	(Xu et al. 2014) (Tjensvoll et al. 2016)
Droplet-based digital PCR	0.005% - 0.01%	CRC Melanoma Breast NSCLC	<i>KRAS</i> <i>BRAF, NRAS</i> <i>PIK3CA</i> <i>EGFR</i>	(Taly et al. 2013) (Tsao et al. 2015) (Oshiro et al. 2015) (Zhu et al. 2015)
BEAMing-Digital PCR	0.01%-1.7%	Breast NSCLC Melanoma	<i>PIK3CA</i> <i>EGFR</i> <i>BRAF, cKIT, NRAS, TERT</i>	(Higgins et al. 2012) (Thress et al. 2015) (Lipson et al. 2014)
Whole genome sequencing	1-5 %	Prostate CRC		(Heitzer et al. 2013) (Diaz et al. 2013)
Whole exome sequencing	1%	Breast, ovarian and lung		(Murtaza et al. 2013)
TAm-Seq	2%	Ovarian and Breast Breast	Gene Panel <i>PIK3CA, TP53</i>	(Forsheew et al. 2012) (Dawson et al. 2013)
Safe-SeqS		CRC	<i>KRAS, BRAF, TP53, SMAD4, PIK3CA, APC</i>	(Bettegowda et al. 2014)
CAPP-Seq	0.02%	NSCLC	Gene panel	(Newman et al. 2014)
Ion-AmpliSeq	0.5%	Breast	Gene Panel	(Rothe et al. 2014)

^a Estimated detectable fraction of mutated alleles (%)

Abbreviations: BEAMing Digital PCR = Beads, Emulsions, Amplification and Magnetics; CAPP-Seq = Cancer personalized profiling by deep sequencing; CAST-PCR = Competitive allele-specific hydrolysis probes (TaqMan); CRC = colorectal carcinoma; NSCLC = non-small cell lung cancer; PCR; COLD-PCR = coamplification at lower denaturation temperature-PCR; *PIK3CA* = phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha gene; PNA-PCR = peptide-nucleic-acid-mediated-polymerase chain reaction clamping; Safe-SeqS = Safe-sequencing system; TAm-Seq = Tagged amplicon deep sequencing; TERT = Telomerase Reverse Transcriptase gene.

Digital PCR (dPCR) based technologies effectively address limitations in earlier PCR based approaches. It separates the sample DNA molecules across a very large number of individual compartments such that each chamber has either one target template or none. This technique was developed by Vogelstein and Kinzler (1999) and has been subsequently optimised using droplet-based (emulsion) or chip-based (nanofluidic) systems (Baker 2012). BEAMing PCR technology (Beads, Emulsions, Amplification, Magnetics) combines emulsion PCR with magnetic beads and flow-cytometry which enable highly sensitive mutational analysis and quantification of ctDNA (Diehl et al. 2006; Richardson and Iglehart 2012). Studies have used dPCR based system to sensitively detect mutations in ctDNA at a very low allelic frequency (0.005% - 0.01%) in various tumour type (Table 1). However, dPCR is expensive, time consuming, require optimisation for each patient, and, like other targeted-based approaches described above, require prior knowledge of specific mutations to be analysed (reviewed in Luke et al. 2014).

The untargeted approach using next generation sequencing (NGS) also known as massively parallel sequencing offer promising potential to identify genome-wide tumour-derived alterations in circulating DNA. NGS-based platforms have been used by different studies to perform whole genome and whole-exome analysis of circulating DNA, and capture known cancer gene panel (Table 1). However, there is a trade-off between increasing the portion of the genome to be sequenced and loss of coverage (depth) which limits the ability to detect the variant of low allelic fraction and differentiate it from a technical artefact (Gagan and Van Allen 2015). Although genome-wide analysis of ctDNA (whole genome and exome sequencing) have promising applications, it is currently expensive and have lower analytical sensitivity (>1%) compared to dPCR-based approaches (Table 1) (Leary et al. 2012; Murtaza et al. 2013; Ma et al. 2015).

On the other hand, sequencing based on gene panel approach using hybrid capture (CAPP-Seq) or amplicon sequencing (TAm-Seq and Safe-SeqS) facilitate low-cost, high throughput and targeted deep sequencing of ctDNA (Forsheew et al. 2012; Newman et al. 2014). In a study by Newman et al. (2014), cancer personalized profiling by deep Sequencing (CAPP-

Seq) was able to detect mutant allelic fraction as low as 0.02% which highlights the high analytical sensitivity of gene panel-based NGS approaches. However, these techniques require advanced bioinformatics platform and are unable to detect translocation and rearrangements without prior knowledge of breakpoint information in case of amplicon-based sequencing (Bratman et al. 2015; Gagan and Van Allen 2015).

Technical aspects

ctDNA is a technically challenging analyte owing to lower concentration of tumour-specific DNA within the background of cfDNA derived from non-tumorous cells. Pre-analytical and analytical issues related to ctDNA measurement hinders consistency and reliability of the results obtained. Studies have shown that concentration of cfDNA is affected by various pre-analytical factors related to blood sampling and processing such as use of different anticoagulants for sample collection, time interval between sample collection and processing, centrifugation conditions and whether serum or plasma is used for ctDNA analysis (reviewed in Jung et al. 2010). The use of serum or plasma as an optimal sampling specimen is controversial (Schwarzenbach et al. 2011). Although the amount of circulating DNA in serum is about 3 to 24 fold higher than those found in plasma (Jung et al. 2003), it is considered a less suitable material because of DNA contamination from leukocyte during the clotting process (Lee et al. 2001). However, a study by Umetani et al. (2006) showed that increased concentration of circulating DNA observed in serum might not be due to extraneous DNA contamination and could occur due to unequal distribution of tumour-related cfDNA during serum separation from whole blood. Nonetheless, plasma should be preferred over the serum as a sampling specimen to obtain reliable results due to a lower level of background wild type extraneous DNA. Different studies have also made similar recommendation (Park et al. 2012; Heitzer et al. 2015). Standardisation and methodological harmonisation of pre-analytical variables are important to achieve reliable, consistent and comparative results.

Analysis of ctDNA using different assay platforms and DNA isolation techniques are also responsible for the inconsistencies in the results obtained by various

studies. As demonstrated in *Table 1*, analytical approaches and assay sensitivity varies between different platforms. A recent study by Thress et al. (2015) compared the ability of different technological platforms to detect EGFR mutation (T790M) from ctDNA. The study has showed that digital PCR-based methods (droplet digital-PCR and BEAMing digital-PCR) were purportedly more sensitive than non-digital platforms (ARMS) for detection of mutant sequences. Additionally, as reviewed by Jung et al. (2010), a large number of alternative protocols for DNA isolation is also responsible for variability in circulating DNA concentration observed by different studies. Therefore, it is imperative to develop consensus on reliable and efficient methods to achieve standardisation.

CLINICAL IMPLEMENTATION OF ctDNA

ctDNA has emerged as a promising biomarker with various potential applications in precision oncology.

Applicability of ctDNA as a diagnostic biomarker

Analysis of tumour-specific mutations as a diagnostic biomarker have been demonstrated by different studies (*Table 2*). Detection of corresponding mutations in various genes including Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*), Tumor Protein P53 (*TP53*) and Adenomatous Polyposis Coli (*APC*) have been found in patients with pancreatic carcinoma (Kinugasa et al. 2015), colorectal cancer (Tie et al. 2015) and ovarian cancer (Forsheew et al. 2012), to name a few (See *Table 2* for selected studies assessing ctDNA in different tumour type). In an exemplary paper, Bettgowda et al. (2014) have demonstrated the ability of ctDNA to detect the tumour in a large cohort of patients with different tumour type. They have shown that detectable levels of ctDNA were found in 49% to 78% of patients with localised tumours. However, as indicated in the same study, patient with stage 1 cancer and neoplasm of CNS (e.g. Glioma) showed a very low level of ctDNA. Moreover, Trombino et al. (2005) found no concordance between *KRAS* mutations found in circulating DNA and tumour tissue from patients with NSCLC. Ramirez et al. (2003), on the other hand, found more mutations in *KRAS* gene in the serum of patient compared to the primary NSCLC.

These contradictory findings question the validity of tumour-specific mutations as a diagnostic biomarker.

Another approach to facilitate cancer diagnostics using ctDNA is detection of epigenetic alterations particularly methylation changes in the circulating DNA. Aberrant DNA methylation in the numerous gene such as *APC*, Glutathione S-Transferase Pi 1 (*GSTP1*), Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*), *p16*, *SEPT9*, O-6-Methylguanine-DNA Methyltransferase (*MGMT*), Retinoic Acid Receptor Beta (*RARβ2*) and Ras Association Domain Family Member 1A (*RASSF1A*) have been analysed in cfDNA extracts (reviewed in Jung et al. 2010; Schwarzenbach et al. 2011). The diagnostic sensitivity of methylation markers in different cancers varies from 2% to 75% using a single methylation marker (Jung et al. 2010). Combining methylation markers remarkably improves the diagnostic sensitivity. As shown in a study by Skvortsova et al. (2006), combining methylation markers *RARβ2* and *RASSF1A* provide 95% diagnostic coverage (sensitivity) for detection of breast cancer. However, Fujiwara et al. (2005), reported cases where no concordance was observed between circulating DNA methylation and corresponding tumour tissue. The authors have speculated that it might be due to undetected pre-cancerous lesions or caused by environmental factors such as smoking. Additionally, the altered methylation patterns in circulating DNA are also age-dependent (Fleischhacker and Schmidt 2007). Therefore, these factors should be taken into account before using methylation status as a tumour-specific biomarker.

Applicability of ctDNA as a prognostic biomarker

Stratification of cancer patients based on clinical prognosis is very important to precisely tailor the treatment according to the need of individual patients. In this regard, ctDNA plays a very important role to stage cancer, monitor tumour burden, predict survival and detect disease recurrence.

Molecular staging of the tumour is one of the reliable predictors of prognosis (Eschrich et al. 2005). Studies have shown a significant correlation between disease stage and the level of tumour-associated genetic alterations found in the circulation (reviewed in

Crowley et al. 2013). A study by Diehl et al. (2005) conclusively demonstrated that the fraction of mutant molecules (*APC*) found in the plasma of patients with CRC were significantly associated with tumour stage. A recent study by Bettegowda et al. (2014) also supports this conclusion and showed that the concentration of ctDNA in the plasma increases with tumour stage across various tumour type. They found that the fraction of patients with detectable ctDNA was 47, 55, 69, and 82% in stage I, II, III, and IV cancers, respectively. However, conflicting results were obtained by some earlier studies in patients with pancreatic and CRC, showing no correlation between *KRAS* gene mutations in circulating DNA and clinicopathological parameters including tumour stage (Yamada et al. 1998; Frattini et al. 2008). Controversies associated with these studies might be due to the limited sample size and selection of appropriate patient cohort, for example, in the above-mentioned study by Frattini et al. (2008), there was considerable variability in certain demographics characteristics of patients such as tumour size and tumour-grade (moderate or poor).

Although ctDNA shows a great promise in staging tumour as described above, it might not be superior to current clinical methods (radiology, histopathology and clinical observation) to stage a tumour. As the concentration of ctDNA depends on the cellular turnover which increase with the volume of the tumour (Schwarzenbach et al. 2011), it might not be effective to stage early-cancer when the tumour volume is significantly low.

On the other, ctDNA has emerged as a superior biomarker to predict survival, determine the risk of recurrence and detect minimal residual disease. As shown in *Table 2*, different studies have demonstrated the remarkable potential of ctDNA to determine prognosis in breast, lung, CRC and ovarian cancer. Especially in the setting of advance-stage, non-resectable tumour, ctDNA offers an effective alternative to identify tumour-specific mutations in plasma that hold prognostic and predictive information. In a study by Nygaard et al. (2013), *KRAS* mutations in plasma were found to be an independent prognostic marker in patients with NSCLC. The authors have suggested that the detection of plasma mutated *KRAS* could serve as an alternative to

invasive tissue biopsy in establishing prognosis in these patients. However earlier study by Camps et al. (2011) using the same technique (qPCR) to detect plasma mutated *KRAS* showed no significant relationship with overall survival and progression-free survival in patients with advance-stage NSCLC. The opposing outcome between these two studies might be due to methodological differences. Both of these used qPCR to detect mutation, but the earlier study by Camps et al. (2011) targeted only two *KRAS* mutations in codon 12, while the later study interrogated six *KRAS* mutations in codon 12 thus resulting in increased detection of plasma mutated *KRAS* (17.5% compared to 8.8% in earlier study). However, the prognostic value and clinical utility of ctDNA in the context of a resectable tumour is very limited. Since the tumour sample is itself available, conventional tests such as histology, immunohistochemistry and molecular analysis can be performed to determine prognosis (Crowley et al. 2013).

Early identification of disease recurrence is important to stratify patients, so that aggressive and multidisciplinary approaches could be adopted in patients with a limited disease which might improve disease control and offer survival benefit (Pagani et al. 2010). In this regard, ctDNA has proved to be a useful, minimally invasive biomarker in setting of adjuvant therapy. In a recent study by Olsson et al. (2015), the eventual clinically detected recurrence (metastasis) was accurately determined by serial monitoring of ctDNA in patients with primary breast cancer who underwent potentially curative surgery. The study also showed that in 86% of the patients, ctDNA-based detection of occult metastasis preceded the clinical diagnosis with a mean lead time of 11 months. However, the current study was limited by small sample size (20 patients) and retrospective study design. Other studies have also highlighted the role of ctDNA as a predictor of poor outcome and showed a consistent relationship between disease recurrence and reappearance of tumour-specific aberrations in circulating DNA in various cancer type (Diehl et al. 2008; Kuhlmann et al. 2012; Kinugasa et al. 2015), also see *Table 2*. Moreover, studies have shown that the ability of ctDNA to predict recurrence were superior to standard biomarker (CEA) (Diehl et al. 2008; Sato et al. 2016).

Table 2. Selected studies of ctDNA detection in various tumour types.

Tumour type	Patients number	Genetic alterations	Source	Analytical Platform	Clinical Utility	Reference
Colorectal Cancer	18	Mutations APC, APC, KRAS, PIK3CA, TP53	Plasma	BEAMing Digital PCR	Prognostic (Tumour burden)	(Diehl et al. 2008)
	53	Mutations in panel of 15 genes	Plasma	Safe-SeqS	Prognostic (Tumour response)	(Tie et al. 2015)
	64	Mutations in KRAS, BRAF, TP53, SMAD4, PIK3CA, APC	Plasma	Safe-SeqS	Diagnostic, Prognostic (Tracking resistance)	(Bettegowda et al. 2014)
	29	Mutations in KRAS, Methylation RASSF2A promoter	Serum	PCR, MS-PCR	Prognostic (Clinical outcome)	(Lefebure et al. 2010)
Breast Cancer	30	Mutations in PIK3CA, TP53, and Structural variation	Plasma	TAm-Seq and digital PCR	Prognostic (Tumour burden)	(Dawson et al. 2013)
	20	Chromosomal rearrangements	Plasma	droplet digital PCR	Prognostic (Poor outcome, eventual clinical recurrence)	(Olsson et al. 2015)
	171	Mutations in <i>ESR1</i>	Plasma	Digital PCR	Predictive (Predict for resistance to Aromatase inhibitors therapy)	(Schiavon et al. 2015)
	72	Mutations in <i>PIK3CA</i>	Plasma and Serum	ARMS-Scorpion, PCR	Predictive (Predict response to PI3K inhibitor)	(Board et al. 2010)
Non-small-cell lung cancer	246	Mutations in <i>KRAS</i>	Plasma	ARMS-qPCR	Prognostic (poor outcome)	(Nygaard et al. 2013)
	803	Mutations in <i>EGFR</i>	Plasma	Allele-specific PCR	Predictive (predict response to Gefitinib treatment)	(Douillard et al. 2014)
	13	Multiple somatic mutations and fusions	Plasma	CAPP-Seq	Diagnostic and Prognostic	(Newman et al. 2014)
Ovarian cancer	38	Mutations in <i>TP53</i> , <i>PTEN</i> , <i>EGFR</i> , <i>BRAF</i> , <i>KRAS</i>	Plasma	TAm-Seq, digital PCR	Prognostic (metastatic relapse)	(Forsheew et al. 2012)
	137	Mutations in <i>TP53</i>	Plasma/serum	PCR	Prognostic (Reduced Survival)	(Swisher et al. 2005)
Pancreatic cancer	121	Mutations in <i>KRAS</i>	Plasma	Safe-SeqS	Diagnostic and Prognostic	(Bettegowda et al. 2014)
	75	Mutations in <i>KRAS</i>	Serum	Droplet digital PCR	Diagnostic and Prognostic (Predicting survival)	(Kinugasa et al. 2015)
	14	Mutations in <i>KRAS</i>	Plasma	PNA-PCR	Prognostic (predict survival)	(Tjensvoll et al. 2016)
Oesophageal cancer	39	Promoter hypermethylation in <i>TP53</i>	Serum	MS-PCR	Diagnostic (screening patients)	(Hibi et al. 2001)
Hepatocellular carcinoma	26	Hypomethylation and copy number aberrations	Plasma	Bisulfite sequencing (WGS)	Diagnostic and Prognostic	(Chan et al. 2013)
Oral squamous-cell carcinoma	20	Microsatellite loci	Serum	PCR	Prognostic	(Kakimoto et al. 2008)

Abbreviations: ARMS-PCR = Amplification-refractory mutation system; BEAMing Digital PCR = Beads, Emulsions, Amplification and Magnetics; CAPP-Seq = Cancer personalized profiling by deep sequencing; *ESR1* = Estrogen Receptor 1; MS-PCR = Methylation specific PCR; PNA-PCR = peptide-nucleic-acid-mediated-polymerase chain reaction clamping; *PTEN* = Phosphatase and Tensin Homolog; Safe-SeqS = Safe-sequencing system; TAm-Seq = Tagged amplicon deep sequencing; WGS = Whole Genome Sequencing

ctDNA is also superior to radiological studies in detecting disease recurrence. A recent study by Tie et al. (2015) showed that early changes in ctDNA in CRC patients receiving chemotherapy predict later radiological response. The resolution limit of radiological imaging studies to detect tumour is a tumour-size of approx. 7–10 mm which contain about 1 billion cells (Francis and Stein 2015). In comparison, tumour containing about 50 million malignant cells releases sufficient DNA which can be detected in circulation (Diaz et al. 2012). Monitoring tumour progression and recurrence requires selection of suitable mutations which are present from the initiation of tumourigenesis and is less responsive to therapeutic interventions (Crowley et al. 2013). However, selection of these candidate mutations is not a simple task because as cancer evolve, it acquires a plethora of somatic mutations (Bardelli et al. 2003), and therefore, detailed knowledge of different key mutations in different cancers is required. Multiplexed mutation detection across a panel of gene holds better clinical utility as a compared to detecting frequently mutated gene (Kidess et al. 2015).

Applicability of ctDNA as a predictive biomarker

The presence or absence of actionable oncogenic mutations in the archived tumour tissue sample is currently being used to guide clinical management using a number of available targeted therapies such as gefitinib, cetuximab, everolimus etc. (reviewed in Tu et al. 2016). As described above, archived tissue specimen does not effectively reflect the genomic landscape of a tumour. These genetic alterations have also been determined in ctDNA which suggests that it could be used as a viable alternative to invasive tissue analysis. In an elegant study by Thierry et al. (2014), authors have compared *KRAS* and *BMRAF* mutations in tumour tissue obtained by routine methods and plasma DNA and demonstrated a very high concordance (96%) between these two methods. Spindler et al. (2012) showed that quantitative analysis of plasma mutated *KRAS* helps in stratification of patients with metastatic CRC, and identify patients who could potentially benefit from anti-*EGFR* therapy.

ctDNA analysis has also emerged as a tool for monitoring therapeutic response and early detection of acquired resistance (Table 2). In a study by Gray et al. (2015), the plasma concentration of *BRAF* and *NRAS* mutations in patients with advanced metastatic melanoma were found to be associated with treatment response. Likewise, a study by Dawson et al. (2013) showed that the trend of serial ctDNA levels correlated with radiologic response to therapy in patients with metastatic breast cancer. These studies highlighted the potential of ctDNA analysis in monitoring tumour dynamics following treatment. Additionally, ctDNA analysis has been used to detect resistance-associated mutations which were previously detected by tumour re-biopsy using invasive clinical procedures. In a study by Zheng et al. (2016), *EGFR* T790M mutation which is associated with acquired resistance to TKI therapy in patients with NSCLC has been demonstrated in ctDNA and has shown to be a minimally invasive alternative to guide clinical management in these patients.

CONCLUSIONS

ctDNA has emerged as a minimally invasive substitute to traditional tumour tissue biopsy. Growing evidence have highlighted the promising role of ctDNA as a prognostic and predictive biomarker and demonstrated that it could be used as a surrogate for tumour tissue to predict the outcome, monitor tumour burden, detect recurrence, and identify genetic determinants for therapy. However, despite great potential of this technique, it has not been approved for routine clinical use.

Many hurdles should be overcome before ctDNA analysis can be adopted into routine clinical practices. One of the major challenges is the lack of standardisation in technical approaches. Inconsistencies are observed in preferable sample type (plasma or serum), sample processing and techniques employed for genotyping ctDNA. Therefore, methodological harmonisation, standardisation and consensus on technical approaches are required to achieve reliable, consistent and comparative results. Another major challenge is related to the specificity of molecular alterations. Individual tumour-derived mutations are never 100% specific for particular cancer and significant overlap

has been observed in different cancers. Thus, ctDNA analysis using a single gene-marker is of limited value and might lead to false-positive results. On this account, ctDNA analysis using a multi-gene panel may increase the test specificity. Additionally, although promising results have been shown by different proof-of-concept studies, prospective, well-designed and adequately powered validation studies in a large cohort and multiple tumour types are required to establish the clinical validity and utility of ctDNA analysis.

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